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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

A COMPARATIVE STUDY OF THE ALDOLASES OF *FASCIOLA HEPATICA* AND *ZYGOCOTYLE LUNATA*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

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JAMES SCOTT McDANIEL

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A COMPARATIVE STUDY OF THE ALDOLASES OF

FASCIOLA HEPATICA AND ZYGOCOTYLE LUNATA

APPROVED BY

DISSERTATION COMMITTEE

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A COMPARATIVE STUDY OF THE ALDOLASES OF *FASCIOLA HEPATICA* AND *ZYGOCOTYLE LUNATA*

CHAPTER I

INTRODUCTION

The carbohydrate metabolism of parasitic helminths has been investigated extensively, and reviews of most of the work are available (von Brand, 1960; Read, 1961; Read and Simmons, 1963). Evidence for the involvement of the Embden-Meyerhof sequence in the primary catabolism of carbohydrate is substantial, and some of the enzymes associated with phosphorylative glycolysis have been characterized. Differences have been demonstrated between host and parasite enzymes as well as between enzymes from different species of parasites. They may differ in kinetic properties (Mansour and Bueding, 1953; Bueding and MacKinnon, 1955a; Bueding and Mansour, 1957; Agosin and Aravena, 1959a), in substrate specificity (Bueding, Ruppender and MacKinnon, 1954; Bueding and MacKinnon, 1955b; Agosin and Aravena, 1959a), in reactions with chemotherapeutic agents (Mansour and Bueding, 1954; Bueding and Mansour, 1957), and in immunochemical reactions (Mansour, Bueding, and Stavitsky, 1954; Bueding and MacKinnon, 1955b; Henion, Mansour, and Bueding, 1955).

Several studies on the characteristics of aldolases of organisms other than helminths have been published. Herbert, *et al.*, (1940) surveyed the physical properties of rabbit muscle aldolase. Warburg and

Christian (1943) studied the properties of crystalline muscle aldolase and aldolase from yeast. The conditions necessary for optimal activity of this enzyme in Clostridium perfringens were determined by Bard and Gunsalus (1950). The aldolase from culture forms of Trypanosoma cruzi was described by Baernstein and Rees (1952). It was also studied in the culture form of Trichomonas vaginalis (Baernstein, 1955). Phifer (1962) compared the aldolases of Aedes aegypti, Anopheles quadrimaculatus, and Culex quinquefasciatus.

Aldolase from helminths has been characterized only for larvae of two species. That from the cysticercus of Taenia crassiceps has a functional pH optimum of 8.9 to 9.0 in tris buffer, a K_m of 6.1×10^{-3} M FDP, and an extended stability at -20 C. It is not inhibited by cations nor are they necessary for optimal functioning, since metal-binding agents do not inhibit. Iodoacetate is not inhibitory to this enzyme, indicating that there are no functional sulfhydryl groups (Phifer, 1958). Larval Trichinella spiralis aldolase functions optimally at about pH 8.5 in tris buffer, and has a K_m of 3×10^{-4}. It is inhibited by cations, but metal and sulfhydryl binding agents do not effect it (Agosin and Aravena, 1959b).

This study concerns the characterization of aldolase in homogenates of two species of trematodes, Fasciola hepatica Linnaeus, 1758, and Zygocotyle lunata (Diesing, 1836) Stunkard, 1917, and compares its activity in the two worms. When possible, comparison with aldolase reported from other sources is made.

CHAPTER II

MATERIALS AND METHODS

Fasciola hepatica of the Texas Gulf Coast strain was obtained from the bile ducts of locally slaughtered cattle and from laboratory maintained infections in albino mice. Flukes from the abattoir were transferred to the laboratory in the saline medium of Dawes (1954). Laboratory infections were terminated between 37 and 42 days.

Zygocotyle lunata was collected from the caeca of naturally infected domestic ducks. Laboratory stocks of this worm were maintained in albino mice for periods of from 28 to 32 days before use.

For experiments, gravid worms with empty guts were selected and washed several times with 0.154 M KCl made alkaline with 8 ml/liter of 0.02 M $KHCO_3$. They were then suspended in an appropriate amount of this medium, and homogenized in an all-glass Potter-Elvehjem homogenizer immersed in crushed ice. Fresh whole homogenate containing approximately 4 mg of wet tissue/ml was used for assay. Comparisons were made between this standard mixture and whole homogenate, centrifuged homogenate, homogenate of flukes with full guts, and whole worms, stored for varying lengths of time at -20 C, and suspended in either 0.85% (w/v) NaCl or 0.154 M KCl. Centrifugation was at approximately 600 x g for 15 minutes in a cold centrifuge head.

Aldolase was determined by a modification of the method of

Sibley and Lehninger (1949) as outlined in Sigma Technical Bulletin No. 750. Additions to the standard mixture were substituted for an equal volume of buffer (0.1 ml). A Bausch and Lomb Spectronic 20 colorimeter was used to measure the color density at a wave length of 540 mμ.

Protein was measured by the Lowry method (Lowry, et al., 1951) using crystalline human serum albumin (National Biochemical Corporation) for comparison.

Enzymatic activity is expressed in micrograms of alkali-labile-phosphate formed per hour per milligram of protein. Where applicable, data were analyzed with Student's "t" test and P values of 0.05 or less were considered significant.

The following abbreviations are used throughout the text: Tris for tris(hydroxymethyl)aminomethane; FDP for fructose diphosphate; EDTA for ethylenediamine tetraacetate; TCA for trichloroacetic acid; ALP for alkali-labile-phosphate; K_m for Michaelis-Menten dissociation constant.

CHAPTER III

RESULTS

To compare aldolase activity in several homogenates, the amount of change produced must be directly proportional to the time allotted. In this work, homogenates were incubated between extremes of 10 and 110 minutes. It was established that the production of trioses is linear for about one hour in both flukes (Fig. 1).

Velocity of simple enzymatic reactions is directly proportional to enzyme concentration, and comparable velocities are obtained only when the enzymes are saturated with substrate. The volume of the trematode homogenates was varied from 0.2 to 1.0 ml in 0.2 ml increments, and incubation was carried out in an excess of substrate. Activity is linear throughout this range of enzyme concentration for both worms (Fig. 2).

In considering the effects of time and enzyme concentration on aldolase activity in the homogenates of F. hepatica and Z. lunata, a standard mixture of one ml of homogenate incubated for 30 minutes was used for all subsequent determinations.

To determine the pH optima of their aldolases, the homogenates of the two worms were subjected to a range of pH from 6.4 to 9.6. Optimal activity is at approximately pH 8.1 for both worms when determined in tris buffer (Fig. 3).

When digestion mixtures are incubated at temperatures ranging between 10 C and 60 C, optimal aldolase activity for F. hepatica is at 40 C, and near 50 C for Z. lunata (Fig. 4).

When F. hepatica homogenates are incubated with concentrations of FDP varying from 1.0 to 50 x 10^{-3} M, maximal activity for aldolase is established at 25 x 10^{-3} M and the apparent Michaelis-Menten dissociation constant is about 1 x 10^{-3} M. For Z. lunata, maximum activity is established at 25 x 10^{-3} M, but the apparent K_m is around 3.0 x 10^{-3} M (Fig. 5).

Experiments conducted to test the stability of whole homogenates, centrifuged homogenates, and homogenates of worms with full guts stored at -20 C, show a fairly rapid decrease in activity for each of them from day to day (Fig. 6). The rate of decrease is comparable for all of them, although that for fresh whole homogenates of worms with full caeca is only about one-half that of worms with empty guts.

The suspending fluid does not appear to be a factor in maintaining stability. The pattern is not greatly different when worms are homogenized in the buffered KCl, 0.85% (w/v) NaCl, or 0.154 M KCl.

Moist whole worms stored at -20 C and homogenized just before use show differing stability patterns. Fasciola hepatica aldolase exhibits a pattern of decrease similar to that of the stored homogenates, while Z. lunata activity shows a steady increase over the time investigated (Fig. 6).

The effect of various substances on the aldolase activity of these two species of trematodes is shown in Table I. A metal-binding agent, EDTA, is without effect. Another metal-complexing agent, cyanide, inhibits about 50% at 1 x 10^{-2} M. Iodoacetate, a known sulfhydryl binder, is inhibitory. Of the various metals tested, none is stimulatory. Mg^{++} has no effect and Mn^{++} inhibits strongly. Fe^{+++} is only slightly inhibitory at 1 x 10^{-2} M

while Ca++ appears more selective in its effect than the other ions. Fasciola hepatica activity is affected by Ca++ only at 1 x 10^{-2} M, while it strongly inhibits Z. lunata activity at 1 x 10^{-4} M. EDTA completely reverses the inhibition of the various metal ions except in the case of Mn++, for which inhibition is increased.

Aldolase activity was determined for F. hepatica from naturally infected cattle and laboratory infected mice, and for Z. lunata from naturally infected domestic ducks and laboratory infected mice (Table II). The average activity of Z. lunata aldolase from both hosts is over two times the activity of F. hepatica from both of its hosts. The aldolase activity in Z. lunata from mice is 1.7 times as great as that in the same species from ducks, while the activity in F. hepatica from cattle is 1.2 times that in individuals from mice.

A recalculation of the maximum activity as milligrams of organic phosphorus per minute per gram weight of tissue as recommended by Meyerhof and Beck (1944) shows that F. hepatica contains 0.44 units of aldolase activity as compared to 0.86 units for Z. lunata. These figures are far below the activity reported for aldolase activity in rabbit skeletal muscle and in C. perfringens, but the activity in these trematodes is well above that recorded for several other organisms (Table III).

Sibley and Lehninger (1949) found an increase in color production in their aldolase determination when hydrazine (the trapping agent) was added after the incubation of homogenates rather than before incubation had begun. They found this to be due to the action of isomerase in the mixture. Baernstein and Rees (1952) verified this effect as a simple qualitative test for isomerase. Experiments performed with F. hepatica and Z. lunata homogenates reveal no isomerase in either worm (Table IV).

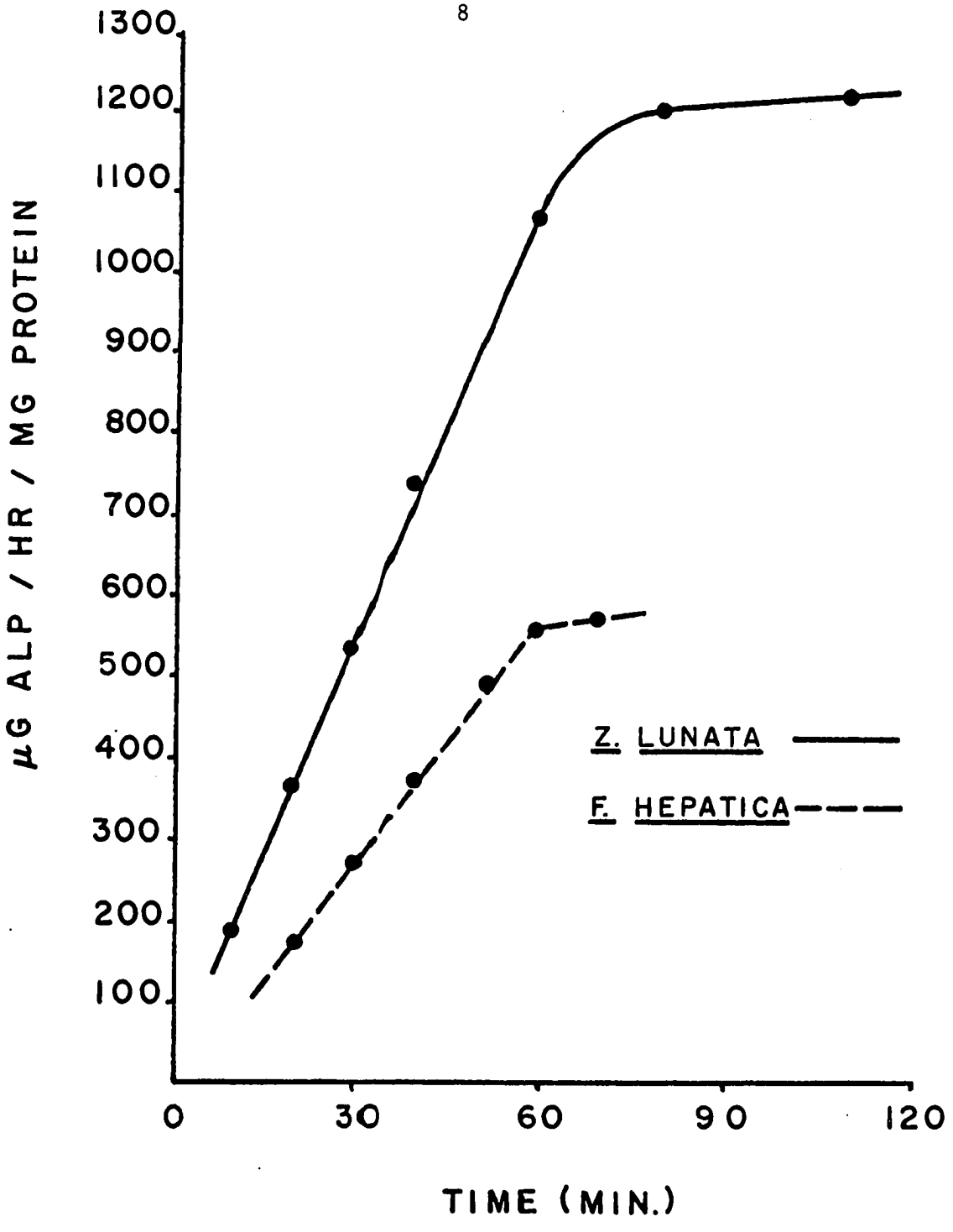

FIGURE 1

THE TIME COURSE OF ALDOLASE ACTIVITY

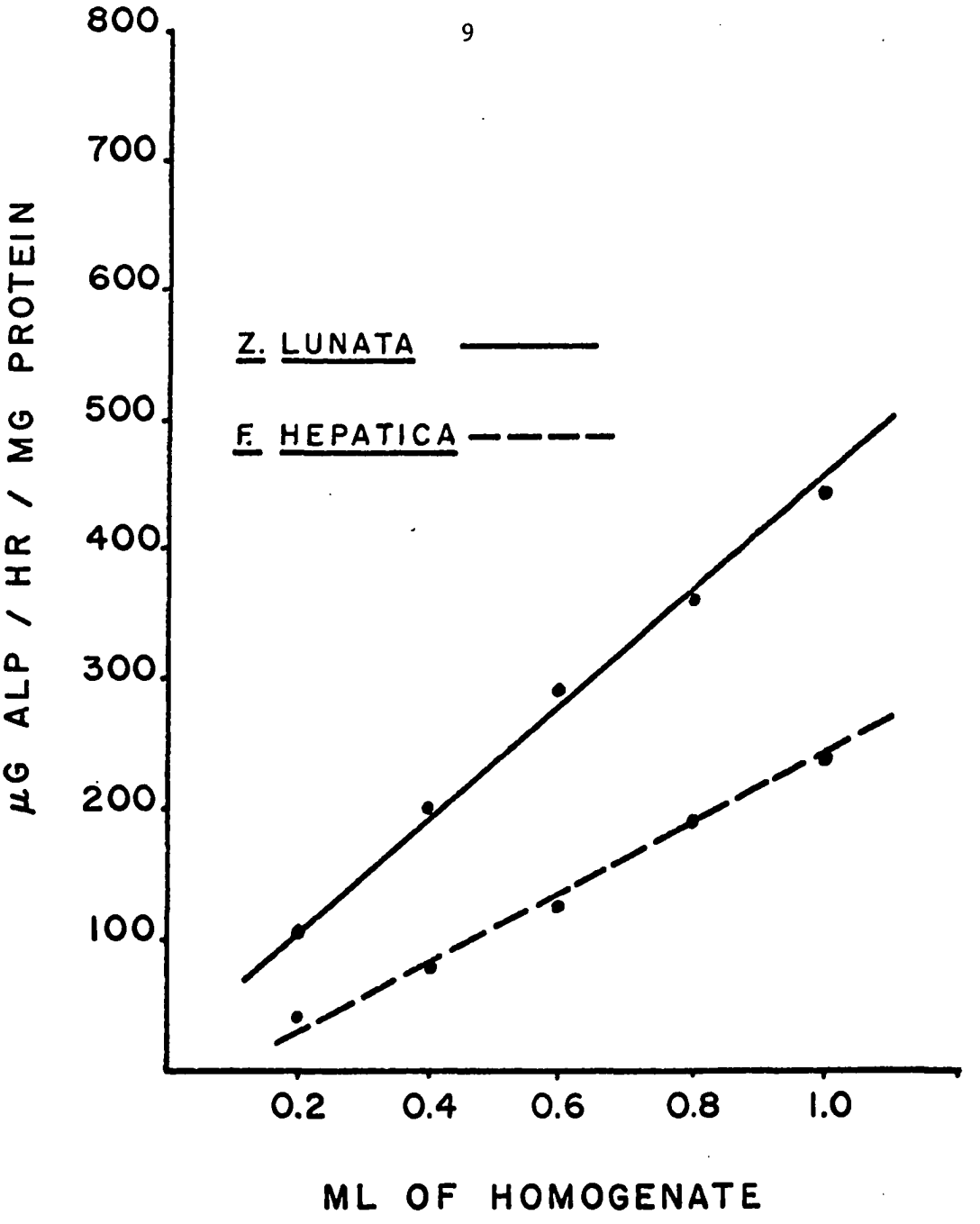

FIGURE 2

EFFECT OF HOMOGENATE CONCENTRATION ON ALDOLASE ACTIVITY

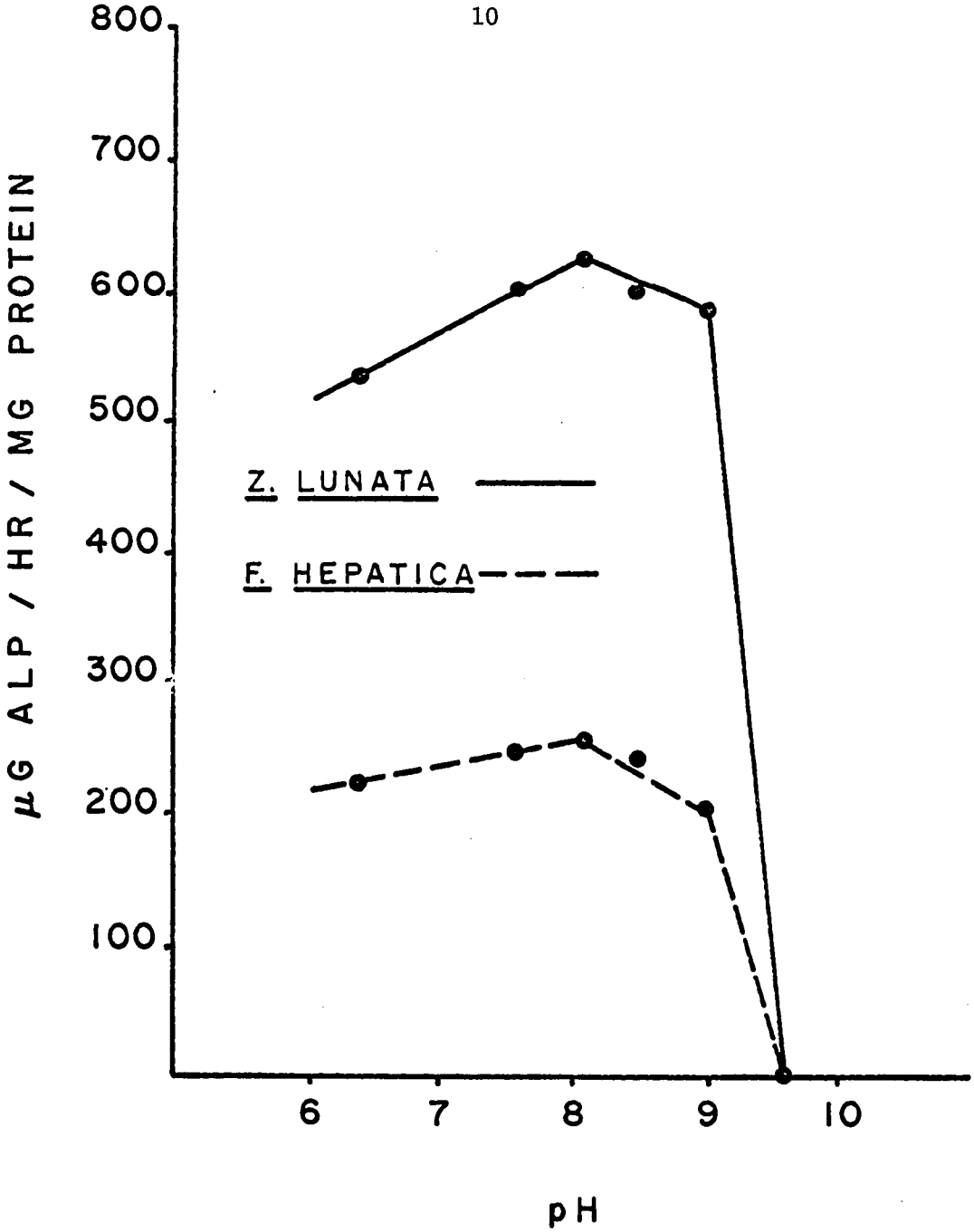

FIGURE 3

THE RELATION OF pH TO ALDOLASE ACTIVITY

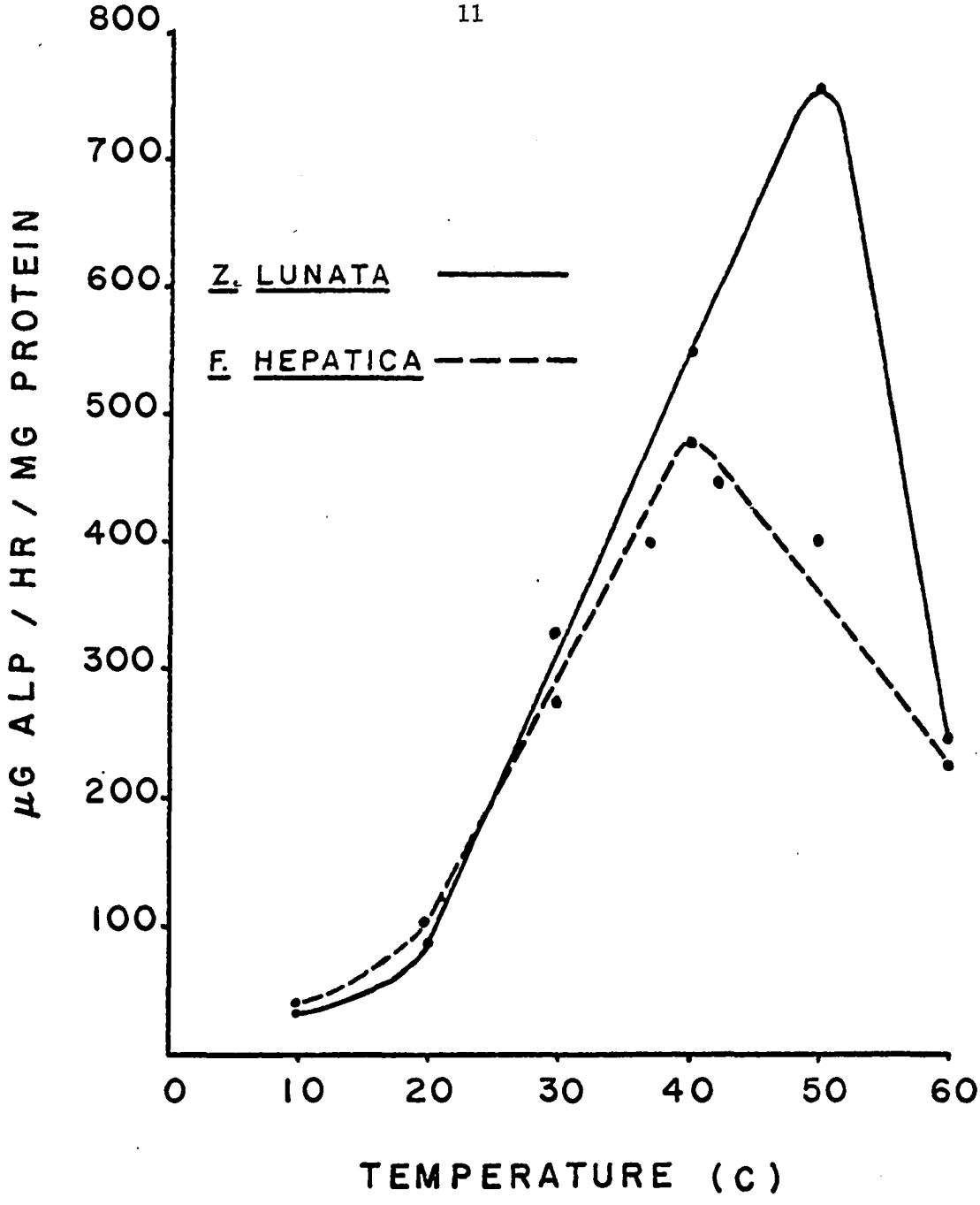

FIGURE 4

EFFECT OF TEMPERATURE ON ALDOLASE ACTIVITY

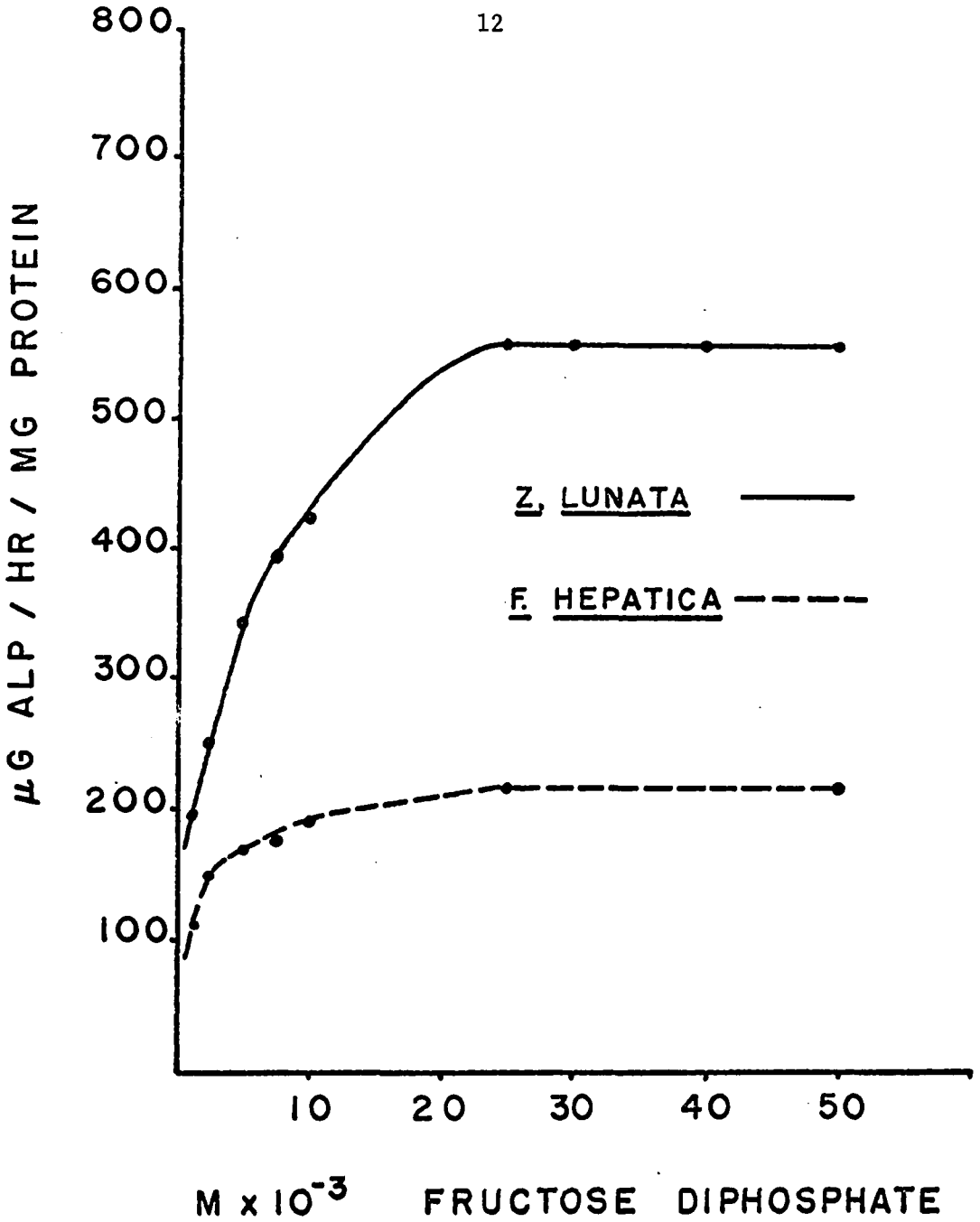

FIGURE 5

EFFECT OF SUBSTRATE CONCENTRATION ON ALDOLASE ACTIVITY

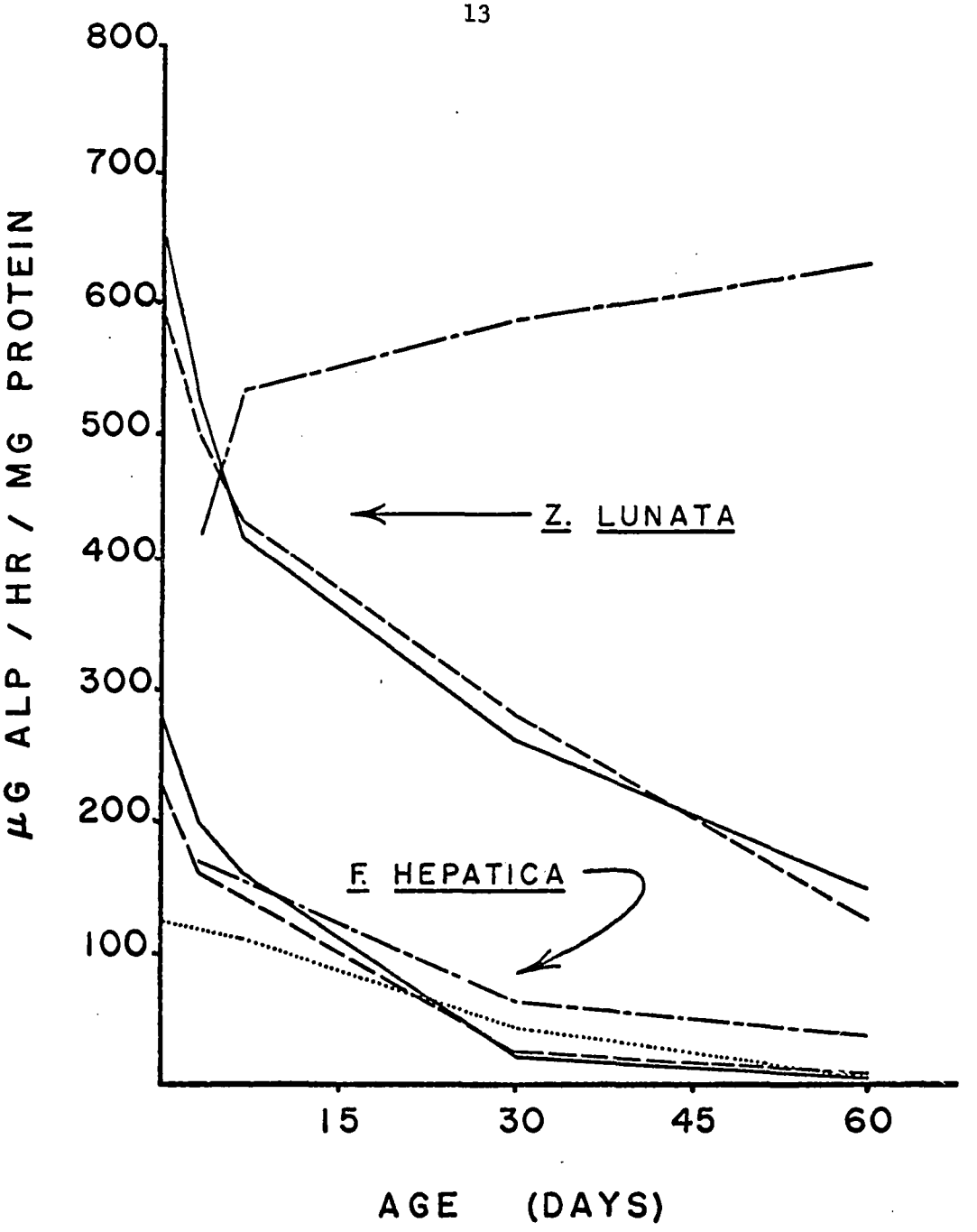

FIGURE 6

STABILITY OF ALDOLASE ACTIVITY

Solid = whole homogenates
Dash = centrifuged homogenates
Long dash = whole worms
Dot = whole homogenate, flukes with full gut

TABLE I

PER CENT INHIBITION OF ALDOLASE ACTIVITY
BY CERTAIN SUBSTANCES

Additions	*Fasciola hepatica*		*Zygocotyle lunata*	
	10^{-2}M	10^{-4}M	10^{-2}M	10^{-4}M
Control	0	0	0	0
EDTA	1	4	0	4
Cyanide	48	0	52	0
Iodoacetate	33	15	22	11
$MgSO_4$	0	0	6	7
EDTA + $MgSO_4$	0	-	0	-
$CaCl_2$	26	0	66	32
EDTA + $CaCl_2$	0	-	9	-
$MnSO_4$	71	43	55	27
EDTA + $MnSO_4$	88	-	83	-
$FeCl_3$	15	3	19	0
EDTA + $FeCl_3$	0	-	4	-

TABLE I

PER CENT INHIBITION OF ALDOLASE ACTIVITY BY CERTAIN SUBSTANCES

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	10^{-2}M	10^{-4}M	10^{-2}M	10^{-4}M
Control	0	0	0	0
EDTA	1	4	0	4
Cyanide	48	0	52	0
Iodoacetate	33	15	22	11
$MgSO_4$	0	0	6	7
EDTA + $MgSO_4$	0	-	0	-
$CaCl_2$	26	0	66	32
EDTA + $CaCl_2$	0	-	9	-
$MnSO_4$	71	43	55	27
EDTA + $MnSO_4$	88	-	83	-
$FeCl_3$	15	3	19	0
EDTA + $FeCl_3$	0	-	4	-

TABLE II

COMPARISON OF *FASCIOLA HEPATICA* AND *ZYGOCOTYLE LUNATA* FROM DIFFERENT HOST SPECIES

Measurement	*Fasciola hepatica*			*Zygocotyle lunata*		
	Cow	Mouse	Average (all runs)	Mouse	Duck	Average (all runs)
Aldolase Activity						
a. µg ALP/hr/mg protein	259.9 (2)*	219.1 (13)	224.5 (15)	573.2 (8)	328.0 (3)	506.3 (11)
b. mg ALP/min/g wet wt.	0.49 (2)	0.40 (14)	0.44 (16)	0.87 (10)	0.82 (3)	0.86 (13)
Total Protein (as % wet tissue)	12.3 (5)	11.4 (10)	11.7 (15)	9.98 (10)	14.6 (7)	11.88 (17)
Average wet weight of the worms (mg)	92.03 (43)	11.13 (44)	44.8 (87)	4.5 (108)	6.6 (55)	5.4 (163)

*Number in parentheses is the number of determinations or worms

TABLE III

COMPARISON OF ALDOLASE ACTIVITY FROM VARIOUS SOURCES

Organism	Activity (mg ALP/min/g wet tissue)	Author
Trichinella spiralis larvae	0.01	Agosin & Aravena, 1959b
Trypanosoma cruzi	0.03	Baernstein & Rees, 1952
Aedes aegypti	0.054*	Phifer, 1962
Culex quinquefasciatus	0.065*	Phifer, 1962
Trypanosoma hippicum	0.07	Harvey, 1949
Anopheles quadrimaculatus	0.10*	Phifer, 1962
Multiceps serialis larvae	0.12*	Esch, 1964
Fasciola hepatica (full gut)	0.27	This paper
Trichomonas vaginalis	0.28	Baernstein, 1955
Fasciola hepatica	0.44	This paper
Multiceps serialis	0.57*	Esch, 1964
Zygocotyle lunata	0.86	This paper
T. vaginalis (activated)	1.60	Baernstein, 1955
Clostridium perfringens	8.0	Bard & Gunsalus, 1950
Rabbit muscle	8.5	Meyerhof, 1951

*calculated from author's data

TABLE IV

THE EFFECT OF HYDRAZINE ON THE AMOUNT OF COLOR PRODUCED (ISOMERASE TEST)

Time Incubated (min.)	*Fasciola hepatica*		*Zygocotyle lunata*	
	Hydrazine before TCA	Hydrazine after TCA	Hydrazine before TCA	Hydrazine after TCA
15	99*	103	119	119
30	195	195	240	240
40	263	268	364	364

* µg ALP/hr/mg protein

CHAPTER IV

DISCUSSION

The carbohydrate metabolism of Fasciola hepatica has been investigated in considerable detail. Evidence for the Embden-Meyerhof sequence is furnished by demonstrations of essential enzymatic steps and identification of necessary chemical intermediates (von Brand, 1952, 1960; Read, 1961). No comparable data exist concerning the mechanisms of anaerobic carbohydrate degradation in Zygocotyle lunata. Demonstration of the existence of an aldolase in Z. lunata in this work, however, is positive evidence for the occurrence of at least a partial Embden-Meyerhof phosphorylative glycolytic scheme, i.e., to the triose stage.

The limits within which linearity of aldolase activity is maintained with respect to time and to concentration of enzyme were determined. In both helminths investigated it was linearly effective for at least one hour at a concentration of 4 mg wet tissue/ml of homogenate (Figs. 1 and 2).

Sibley and Lehninger (1949) reported a pH optimum of 8.5 to 9.0 for rabbit muscle aldolase activity, and Phifer (1958) reported the same for Taenia crassiceps larvae. The optimum pH for aldolase activity in Trichinella spiralis larvae is 8.5 (Agosin and Aravena, 1959b). Phifer (1962) showed that mosquito aldolases vary in optimal activity between pH 7.4 and 8.2. All of these studies involved the use of tris buffer. My studies, also utilizing tris, show that these trematode homogenate aldolases function

optimally near pH 8.1. Studies using other buffer systems tend to demonstrate slightly lower pH optima. The pH optimum for Clostridium perfringens aldolase activity is 7.5 (Bard and Gunsalus, 1950); for Trypanosoma cruzi, 7.3 (Baernstein and Rees, 1952); and for Trichomonas vaginalis, 7.0 (Baernstein, 1955). The pH optima of the aldolase systems of tapeworms, trematodes, and nematodes seem to fall within a relatively small range, viz. 8.1 to 9.0. To compare the pH optima of mammals, helminths, and insects with those of protozoa and bacteria, tris buffer would have to be used in each case. Not only do different buffers affect the activity of an enzyme to different degrees, but tris may act as a catalyst in splitting substrate to trioses in alkaline solution. Furthermore, tris may activate aldolase at high pH's (Dounce, Barnett, and Beyer, 1950).

The effect of temperature is interesting in that Z. lunata aldolase was optimally active at about 50 C, while that in F. hepatica functioned optimally at 40 C (Fig. 4). Taylor, et al.,(1948) found that serum albumin protects aldolase from inactivation on dilution, and thus allows a higher operating temperature. Dounce, et al., (1950) found the optimum temperature for muscle homogenate aldolase to be 35 C, and for that in liver homogenate to be 45 C. They postulated that the difference between them might be due to the lower dilution of liver homogenate used, a factor which permitted protective action against denaturation by proteins in the homogenate. Phifer (1962) accepted this "protective protein" hypothesis to account for differences in temperature optima for aldolase activity among three species of mosquitoes. In this study when the same wet weight of tissue from the two flukes was compared, a statistically significant difference in the amounts of protein in the homogenates of worms taken from mice was demonstrated (Table II). Fasciola hepatica, however, shows the greatest quantity of

protein, and exhibits the lowest temperature optimum. It would appear that the different temperature optima cannot be ascribed to the protective action of homogenate proteins.

In a Michaelis-Menten type of plot (Fig. 5), the K_m is a rough measure of the affinity between enzyme and substrate. The lower the value of K_m, i.e., the steeper the slope of the curve, the greater the approximate affinity. The lowest value reported is 3×10^{-4} M for T. spiralis larvae (Agosin and Aravena, 1959b). Phifer (1958) reported the constant for T. crassiceps larvae as 6.1×10^{-3} M. Baernstein (1955) found the apparent K_m for aldolase in T. vaginalis to be 1.2×10^{-3} M, agreeing with the findings of Baernstein and Rees (1952) for T. cruzi culture, and Bard and Gunsalus (1950) for C. perfringens. The constants for three species of mosquitoes ranged between 3.2×10^{-3} M and 3.6×10^{-3} M (Phifer, 1962). Dounce and Beyer (1948) reported a constant of 9×10^{-3} M for rabbit muscle aldolase. K_m values for FDP of 1×10^{-3} M (F. hepatica) and 3×10^{-3} M (Z. lunata) place these two species of trematodes intermediate between nematodes and tapeworms.

A rapid decline on aging is reported for aldolase activity in C. perfringens (Bard and Gunsalus, 1950), T. cruzi (Baernstein and Rees, 1952), and T. vaginalis (Baernstein, 1955), and the suspending fluid was found to be a factor in the stability of the enzyme in T. cruzi and T. vaginalis. Taenia crassiceps aldolase was found to be considerably more stable with no loss of activity indicated for up to three months at -20 C (Phifer, 1958). These investigators also found centrifuged homogenate to have an activity similar to that of the whole homogenate.

Aldolase activity in F. hepatica and Z. lunata does not decline as rapidly as that in C. perfringens, and the enzymes are not as stable as

that in T. crassiceps. Both trematode homogenates lose activity at about the same rate for the first week, but the rate of drop in F. hepatica from week one through the fourth week is over twice that in Z. lunata (Fig. 6). The supernatant from the centrifuged homogenates gives approximately the same activity as the whole homogenates, and it loses activity at about the same rate (Fig. 6). Evidently the enzymes are soluble and probably not particulate, and the sediments do not contain a labilizing or activating factor. No differences in activity were in evidence among homogenates suspended in buffered or unbuffered KCl or NaCl. The activity in fresh homogenates of F. hepatica with full caeca is considerably lower (50%) than that of the other homogenates, but the rate of decline on aging is not appreciably changed (Fig. 6). The total protein content of the worms with full caeca is about 14% greater than that of worms with empty guts, but this alone does not account for such a large variation in activity. This suggests a partial nonprogressive inhibition of the aldolase activity by substances or secretions in the worms' caeca. The nature of the dark pigmented materials seen in the caeca of feeding flukes is not known, but it is certainly not derived from blood as had long been suspected. These flukes feed on the hyperplastic epithelium of the host's bile duct (Dawes and Hughes, 1964). The cells of the worm's caecal epithelium pass through a series of events that indicates a secretory and absorptive cycle (Dawes and Hughes, 1964; Thorsell and Björkman, 1965). However, Mansour (1959) observed that the rate of glucose uptake in F. hepatica is the same in worms with or without a ligature closing off their "digestive" tracts. This indicates that neither the absorption of glucose, nor the excretion of metabolic products is carried out through the gut.

Aged whole individuals of F. hepatica and Z. lunata do not show the

same aldolase stability patterns. The decrease of activity in whole F. hepatica is similar to that for stored homogenates, but whole Z. lunata exhibit a steady rise in activity over the time observed (Fig. 6). The reasons for this increase with aging are not readily apparent, but, as stated previously, F. hepatica tissues contain more protein than those of Z. lunata per equivalent amounts of wet tissue. Evidently the increase cannot be attributed to lower dilution of temperature-protective proteins in these worms.

Two types of aldolase have been described based on inhibition by metal-binding agents. Aldolases of yeast (Warburg and Christian, 1943), C. perfringens (Bard and Gunsalus, 1950), Aspergillus niger (Jagannathan and Singh, 1954), and T. vaginalis (Baernstein, 1955) are inhibited by these complexing agents. The aldolases of muscle (Warburg and Christian, 1943), pea (Stumpf, 1948), T. cruzi (Baernstein and Rees, 1952), T. crassiceps (Phifer, 1958), T. spiralis (Agosin and Aravena, 1959b), and Aedes aegypti, Anopheles quadrimaculatus, and Culex quinquefasciatus (Phifer, 1962) are not inhibited by such agents. The interpretation of these experiments is still in doubt since only the muscle enzyme has been crystallized. The enzymes of C. perfringens and A. niger are believed to be metalo-aldolases (Bard and Gunsalus, 1950; Jagannathan and Singh, 1954). Warburg and Christian (1943) suggested that metals may remove a natural inhibitor present in the impure preparations. The mosquito homogenate aldolases investigated by Phifer (1962) are activated by the chelating agent, EDTA, and inhibited by cations. It would appear that a significant level of inhibitory cations is present in them.

Homogenate aldolases of F. hepatica and Z. lunata are not affected by EDTA, nor by cyanide except at high concentrations (Table I). Trapping of triose phosphates by cyanide at a concentration of 0.01 M would account

for the apparent loss of activity because the cyanhydrin of triose phosphate cannot be split again (Meyerhof, 1951). The various metals tested were selective in their effects, but none were stimulatory (Table I). Mg++ was without effect. Mn++ inhibited strongly. Ferric ions caused slight inhibition at high concentrations. Ca++ inhibited both aldolases, but only at high concentrations for F. hepatica. EDTA completely reversed the effects of the metal ions, except for Mn++, in which case the inhibition is actually increased. Iodoacetate was inhibitory, so it would appear that these aldolases have functional sulfhydryl groups (Table I).

Fasciola hepatica is notorious as a ubiquitous parasite of certain herbivorous mammals (Cheng, 1964). That it is not equally adapted to all of the hosts in which it may occur is widely substantiated. Infection rate, worm burden, worm development and fecundity, and pathogenicity, are known to vary greatly among the various hosts (Dawes and Hughes, 1964).

The principal hosts of Z. lunata are water birds (Cheng, 1964), although it has been reported in natural and experimental infections from ruminants and rodents (Willey, 1941). Very little is known about its physiology. Growth and development differences have been noted in worms from ducks and rats (Willey, 1941). Bacha (1959, 1964) observed both a natural resistance and the development of an age resistance in laboratory rats, and that an initial infection causes a decrease in both size and numbers of worms from a challenging infection. Zygocotyle lunata is usually said to be nonpathogenic (Cheng, 1964), however, obvious physical damage is incurred at the site of attachment of the strong ventral sucker in ducks, rats, and mice, and the caeca of infected hosts do not have the same appearance as those of uninfected animals (personal observations).

Large differences in aldolase activity were noted for both F.

hepatica and Z. lunata when grown in different hosts (Table II). The absolute age of worms from natural infections could not be determined, but all used in experiments were producing eggs and, therefore, mature by definition. Further, chemical and physical measurements indicate that these differences cannot be explained on the basis of worm age alone (Table II). Zygocotyle lunata aldolase activity is greatest in worms from mice although these worms were smaller and contained less protein than those from ducks. Activity shown by a single experiment with an old (90 day) infection in a rat (593 μg ALP/hr/mg protein) compared favorably with that in mice, but the worms were twice the size of those taken from mice (8.3 vs 4.5 mg wet wt) and their protein content (8.6% wet wt) was below the average for worms taken from both the other hosts. It should not be assumed that aldolase activity would increase linearly with age and weight throughout the life of the worm, but the agreement of data from rodents would indicate that worms from ducks simply exhibit less aldolase activity than those grown in mice or rats.

The rate of action of aldolase is markedly reduced in Schistosoma mansoni by even a small decrease in its substrate concentration (Bueding and Mansour, 1957). If aldolase is rate-limiting in Z. lunata also, we might conclude that worms from ducks are adapted for a slower rate of carbohydrate utilization than worms from rodents. This could relate to the kind of usable carbohydrate present in the environment because induction theory would predict that enzymatic pathways of lesser importance to an organism would not be maintained at activity levels in excess of need.

Aldolase activity in F. hepatica from cattle is also demonstrably different from that in mice (Table II). The results are not inconsistent with worm size, although to accept this would mean accepting increased activity in older worms without a concomitant increase in body protein.

If aldolase activity alone is used as a measure of the compatibility of the parasite and the host, then it might be said that F. hepatica has its best relationship with the cow and Z. lunata with the mouse, where activity levels are greatest. It can be seen from the discussion above, however, that an increased aldolase activity taken without reference to other metabolic information, is not a sure explanation of the compatibility of a particular host-parasite relationship.

Calculation of aldolase activity by the method suggested by Meyerhof and Beck (1944) places F. hepatica and Z. lunata in a somewhat median position among other organisms that have been investigated (Table III). The use of hydrazine as a trapping agent in the Sibley and Lehninger aldolase determination (1949) probably invalidates a strict comparison with values obtained without it, but several methods for the determination of aldolase using other trapping agents give values that agree fairly well (Dounce, et al, 1950; Meyerhof, 1951).

Two helminths for which aldolase activity has been determined are not included in Table III because the arbitrary units of measurement used by the investigators make comparison with the Meyerhof units subject to considerable uncertainty. Taenia crassiceps larvae is one of these (Phifer, 1958). Normal activity is not stated, but data supplied indicate it to be about 13.3 μg ALP/hr/mg dry wt. If we assume dry weight to be 25% of the wet weight as indicated by data from other helminths (Fairbairn, et al, 1961), then T. crassiceps activity would appear to be 0.055 mg ALP/min/g wet wt. Bueding and Mansour (1957) determined S. mansoni aldolase activity to be 0.17 μmoles FDP used/mg protein/min at 25 C. If we assume that temperature effects S. mansoni aldolase in the same manner as the enzymes of the worms used in my experiments, the activity would be approx-

imately 2.29 mg ALP/min/g wet wt at 37 C.

The activities of the adult helminth aldolases investigated fall within a relatively small range, and they are considerably higher than activities recorded for larval worms. The larvae are not closely grouped, but tapeworms exhibit 5 to 10 times the activity of the single nematode investigated, T. spiralis. Esch (1964) is the only worker to have compared aldolase activity in the adult and larva of the same species. Adult Multiceps serialis aldolase has 5 times the activity of that of its coenurus, and is intermediate between the trematodes of the present study. This is consistent with the observations between species, and indicates that larval activity cannot be assumed to reflect adult biochemistry and physiology.

Baernstein and Rees (1952) applied data gathered by Sibley and Lehninger (1949) in developing a simple indirect method for the qualitative determination of triose phosphate isomerase. It is this enzyme that catalyzes the interconversion of the two triose phosphates produced from FDP degradation by aldolase. The experiments showed that dihydroxyacetone gave about three times as much color as glyceraldehyde on a molar basis. If homogenates are incubated in the presence of hydrazine, the trapping agent, the trioses are immediately bound. If hydrazine is added after the reaction has been terminated, a change in color should reflect the action of isomerase. The equilibrium constant of the isomerization favors the ketotriose, although glyceraldehyde phosphate is the compound which undergoes the subsequent reactions of the glycolytic pathway (Cantarow and Schepartz, 1957). However, the presence of isomerase assures the eventual utilization of both triose moieties. Fasciola hepatica and Z. lunata homogenates definitely show no isomerase by this indirect method (Table IV).

It is frequently stated that parasitic animals are less efficient metabolically than many free-living ones since, typically, carbohydrate is incompletely metabolized, leaving considerable potential energy in the end product molecules. The apparent absence of isomerase in these trematodes is consistent with this hypothesis. However, Read (1961) has pointed out that maintaining a metabolic system which extracts the largest practical amounts of energy per molecule of substrate has a high energy cost. Parasites do not have the physiological problem of obtaining an energy source, so a simplification of catabolic reactions might easily occur. Process thermodynamics would predict so, because as the number of processes decreases the ratio of real work output per unit of theoretical work increases.

The present study constitutes the first characterization of the fructoaldolase enzyme from homogenates of adult helminths, and the first characterizations among the digenetic trematodes. Only aldolases from a larval cestode, T. crassiceps, and a larval nematode, T. spiralis, have been characterized previously (Phifer, 1958; Agosin and Aravena, 1959b). The four aldolases investigated vary in temperature and pH optima, stability, and affinity for substrate (K_m). The aldolases from the larval cestode and nematode do not appear to contain essential sulfhydryl groups, although such groups are apparently present in the aldolases from adult trematodes. Metal activation is not demonstrable in helminth aldolases, but metal ion inhibition is indicated in three instances. Only the T. crassiceps aldolase differs in this regard. In general, the physical characteristics of the aldolases in the two trematode genera are similar, while the two aldolases from larvae differ to some extent from the enzymes of the adults. Such differences might be expected on the basis of taxonomic relationships.

CHAPTER V

SUMMARY

1. Homogenates of Fasciola hepatica and Zygocotyle lunata contain an active aldolase. The demonstration of this enzyme in Z. lunata is the first indication of the occurrence of the Embden-Meyerhof system in this organism.

2. The limits have been determined within which linearity of activity with respect to time and to concentration of enzyme exist.

3. The optimum pH was 8.1 in tris buffer.

4. The optimum temperature for activity was 40 C for F. hepatica aldolase and near 50 C for Z. lunata.

5. The Michaelis-Menten constant was found to be 1.0×10^{-3} M FDP for F. hepatica and 3.0×10^{-3} M for Z. lunata.

6. Both aldolases were unstable when stored at -20 C in various suspending fluids. They evidently are soluble enzymes and the homogenate sediments do not contain an activating or labilizing factor. A partial non-progressive denaturation of the F. hepatica enzyme by substances or secretions in the caeca was indicated.

7. EDTA and cyanide had no effect on the aldolase activity in these animals and lend no evidence that these are metal-activated aldolases. Of the metal ions tested, Mg^{++} had no effect, and Fe^{+++}, Mn^{++} and Ca^{++} were inhibitory to varying degrees. The inhibition can be reversed

with EDTA, except in the case of Mn^{++}.

8. Iodoacetate was inhibitory, indicating functional sulfhydryl groups were present.

9. Activity was demonstrably different between worms grown in different hosts. The variation did not appear to relate directly to size or age of the worms.

10. Aldolase activity per gram wet weight of organisms was equivalent to 0.44 Meyerhof units for F. hepatica and 0.86 units for Z. lunata. This indicates that these worms have very active aldolases and that they probably make considerable use of carbohydrate substrates.

11. Indirect evidence for the absence of triose phosphate isomerase in these worms was presented.

12. This study is the first such investigation on adult helminths, and the first characterizations of aldolase among the digenetic trematodes. The physical characteristics of the aldolases are similar in the two genera, but differ in small degree. Such differences might be expected on the basis of taxonomic relationships.

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